

UNCLASSIFIED

AD 4 3 9 3 1 3

DEFENSE DOCUMENTATION CENTER

FOR

SCIENTIFIC AND TECHNICAL INFORMATION

CAMERON STATION, ALEXANDRIA, VIRGINIA



UNCLASSIFIED

NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.

64-13

M79-61-80

LS

(9)
15
439313

Semi-annual ~~no.~~ REPORT ON no. 1, 1 Nov 62 - 30 Apr 63,

CONTRACT NO DA ~~X92-557-FEC-35673~~

INCLUSIVE DATES ~~1 Nov. 1962~~

~~to 30 April 1963~~

AD No.
DDC FILE COPY

SUBJECT OF INVESTIGATION

6) STUDIES ON THE MODE
OF
ACTION
OF
ANTIBACTERIAL DRUGS.

RESPONSIBLE INVESTIGATOR

10) Dr. Katsuhiko Tago.

Assist. Chief, Tuberculosis Section
Kitasato Institute
~~Tokyo~~, (Japan).

198810

AT

U.S. Army Research & Development Group (9984) (Far East)

Office of the Chief of Research and Development

United States Army

APO 343

439313

12 P \$1.60

D-I-S-T-R-I-B-U-T-I-O-N

The distribution of this report as made by USA R&D Op (FE)
is as follows:

Army Research Office, 3045 Columbia Pike, Arlington 4,
Virginia. ATTN: Chief Research Programs Office

Army Attache, American Embassy, Tokyo, Japan

(1)

Semi-annual No. 1 REPORT ON
CONTRACT NO LA - 92-557-FEC-35673
INCLUSIVE DATES 1 Nov. 1962 30 April 1963

SUBJECT OF INVESTIGATION

STUDIES ON THE MODE
OF
ACTION
OF
ANTIBACTERIAL DRUGS

RESPONSIBLE INVESTIGATOR

Dr. Katsuhiko Tago
Assist. Chief, Tuberculosis Section
Kitasato Institute
Tokyo, Japan

1. Preparation of Cl⁴-leucomycin by fermentation using radio-active precursors.

a. Materials and Methods.

(1) Strain used: *Streptomyces kitasatoensis* was used in these studies. The strain was transferred to slants of either leucomycin medium or starch synthetic medium to which 2% agar was added. The slant cultures were incubated 3 to 7 days. The spores were gently scraped from the culture surface to form a spore inoculum.

(2) Medium used: The following two media were used throughout the studies.

(a) Leucomycin medium.

Glucose	4.0g
NaCl	0.5g
K ₂ HPO ₄	0.1g
(NH ₃) ₂ SO ₄	0.3g
C.S.L.	0.5g
Urea	0.05g
Soi-been powder	2.0g
Yeast extract	0.5g
CaCO ₃	0.3g
Aq. dest.	100 ml.

(b) Starch-synthetic medium.

Soluble starch	0.2g
K ₂ HPO ₄	0.05g
MgSO ₄	0.02g
CaCl ₂	0.02g
NANO ₃	0.005g
Asparagin	0.005g
Fe ₂ (SO ₄) ₃	0.001g
Aq. dest.	100 ml.

Adjust pH 7.4. Agar is added 2.0% in solid medium.

- (3) Method of fermentation: 100 ml. of starch medium was distributed to 250 ml. Erlenmeyer flasks and inoculated 1.0 ml. of 48 hrs. culture of S. kitasatoensis in leucomycin media. Labeled precursors were added to the medium either as solid prior to sterilization or as aqueous solution which had been passed through an ultrafine bacteriological filter and added after sterilization. The fermentation was carried out at 30°C on a shaker.
- (4) Isolation of leucomycin: After filtration of mycelium, the culture filtrate was extracted twice with 50 ml. of ether which was evaporated in vacuo. Dried leucomycin was solved with 1.0 ml. of ether and diluted with adequate amount of aq. dest.
- (5) Estimation of radio-activity: 1.0 ml. of solution of leucomycin was distributed to aluminum cup and vacuum-dried. The ¹⁴C content of dried preparation was estimated by use of 'Riken' radiation counter model RSC-SB.

b. Result

- (1) Fig. 1 shows the relationship between pH value of culture filtrates and incubation period. All media showed the reversal of pH value on the fourth day of the incubation. Among the media tested, leucomycin medium showed the most significant reversal and starch media inoculated spores of S. kitasatoensis slightest reversal. Production of leucomycin in media was paralleled to the reversal of media. Contents of leucomycin in leucomycin-medium was 40 mcg. per ml. and the lowest starch medium inoculated spores was 5 mcg per ml. The optimal concentration of soluble starch in synthetic medium was 0.2 g per liter.
- (2) Twenty hours after inoculation of S. Kitasatoensis in 100 ml. of starch-synthetic medium, 0.1 mc. of Cl^{14} -sodium acetate or Cl^{14} -Starch-U were added as the precursor. Three days after addition of them, radioactive leucomycin was extracted from the culture filtrate. Cl^{14} -acetate-leucomycin showed very high specific activity, 208,000 cpm per μ mol, whereas Cl^{14} -starch leucomycin was only 260 cpm per μ mol.

2. Fractionation of Staphylococcal cells treated with radioactive leucomycin.

a. Materials and Methods.

- (1) Cold TCA fraction: All low-molecular weight compounds soluble in 5% (w/v) teichloroacetic acid are contained. The organism was suspended in 2 ml. cold water, added 0.5 ml. cold 25% (w/v) TCA; after 10 min., centrifuged at 4,000 g for 5 min., and decanted extract.
- (2) Aqueous ethanol-soluble fraction: Ethanol-soluble 'protein' and lipid are contained. The residue was suspended in 2.5 ml. 75% (w/v) ethanol in water; after 10 min. at room temperature centrifuged (4,000 g 10 min.) and decanted extract.
- (3) Hot TCA fraction: Breakdown products of nucleic acid and teichoic acid. The residue was suspended in 2.5 ml. 5% (w/v) TCA, heated 6 min. at 90°C, cooled, centrifuged (4,000g, 10 min.) and decanted extract.
- (4) Trypsin-solubilized: Trypsin-degraded proteins. The residue was suspended in 0.95 ml. 0.05 N-NH₄CO₃ containing 0.005 N-NH₄OH:0.05 ml. of solution containing 1 mg. crystalline trypsin per ml. was added. Incubated 2 hrs. at 37°C or until digestion was complete. Centrifuged (4,000 g, 10 min.) and decanted extract.
- (5) Residue: Mucopeptide of wall. The residue was suspended in 1.0 ml. of water.

b. Results.

Staphylococcal cells treated with Cl^{14} -leucomycin were fractionated by a modified method of Park et al. and radio activity of each fraction was estimated. As shown in Table 2, the high radio activity were revealed in the trypsin digested protein fraction and cell wall mucopeptide fraction, whereas low radio activity were found from ethanol soluble lipid fraction and nucleic acid fraction.

Table 1. Fractionation of Staphylococcus aureus treated with Cl^{14} -leucomycin.

Fraction	Contents of fraction	cpm
1. Cold TCA	All low-molecular weight compounds soluble in 5% (w/v) TCA	35.0
2. Aqueous ethanol soluble	Ethanol-soluble 'protein' and lipid	11.5
3. Hot TCA	Breakdown products of nucleic acid and teichoic acid.	0
4. Trypsin-solubilized	Trypsin-degraded protein	52.5
5. Residue	Mucopeptide of wall	70.0

3. Preparation of ribosomes from staphylococcal cells treated with radioactive leucomycin.

a. Materials and Methods

Bacterial extracts were prepared by grinding washed whole cells with alumina. The alumina and cells (3:1) were homogeneously mixed in an ice cold mortar. To the white paste-like material a volume of extracting solvent was added equal to the wet weight of cells. The solvent contained: Tris buffer (0.1M pH 7.0); MgCl₂ (7 x 10⁻³M); and KCl (6 x 10⁻²M). The mixture of solvent, cells and alumina was vigorously ground for 5 min. Precipitable material was removed by centrifugation (10 min, 10,000g). The precipitate was re-extracted two more times as before, except 2 volumes of extracting solvent were used each time; the supernatants of all centrifugations were then combined and recentrifuged (120 min, 105,000g). The radiation count of the precipitate was performed as mentioned before.

b. Results

Radioactivity of C¹⁴-sodium acetate treated leucomycin was 25 cpm per mg N and that of C¹⁴-starch-leucomycin treated was 15 cpm per mg N. For the estimation of such a low radioactivity, it will be necessary to use a gas-flow windowless radiation-counter.

4. Preparation of C¹⁴-leucomycin by biological method.

a. Materials and methods.

A trained female mongrel dog was anesthetized with an intravenous injection of sodium pental, the cystic duct was tied off with braided silk through a medial abdominal incision. The inner part of a lucite Thomas duodenal fistula was placed opposite the duodenal papilla at which the common bile duct opens; The outer part will be inserted in a lateral abdominal incision about one inch to the right of the middle line and one inch from the last rib. Leucomycin tartrate, which assayed 1,010 mcg. of leucomycin activity per. mg., was administrated intravenously in a dose of 150 mg. per kg. body weight. Bile collected was made for the subsequent six hours by cannulation of the common bile duct with the dog lying at rest. Bile was extracted with five equal volume of chloroform and the green chloroform solution was washed with water, dried over anhydrous sodium sulfate and evaporated to dryness under vacuum.

b. Results.

About 80 mg. of light yellow powder, which is assumed de-N-methyl-leucomycin was extracted with chloroform from the dog bile. The natibacterial activity of this powder was 200 mcg per mg. Seventy six mg. of this powder was methylated with CH₃J in methanol and 28 mg solid material was obtained. But this material was assayed the equivalent of only 250 mcg leucomycin per mg. As the amount of the sample was too little, the completion of methylation was not clear, but from its low antibacterial activity, it might not be true leucomycin. This fact will be clarified when C¹⁴-methyl-jodid is used in our future experiment.

5. Radioautography

Escherichia coli strain K-12 and Staphylococcus aureus FDA 200-P were employed for this experiment. The organisms were cultured in synthetic media containing all the essential amino acids, purines, pyrimidines, vitamins, glucose as energy source and other minerals. Before the addition of labelled compound, logarithmic growth had been maintained by shaking at 37°C for several hours. The organisms were harvested by centrifugation, washed once with 0.1 M phosphate buffer pH 7.2 and resuspended in fresh media containing 8-14C-adenine sulfate instead of unlabelled adenine at the concentration of 0.21 micro mol/ml. Then, the incubation was continued in a water bath at 37°C with shaking. One ml of samples were taken at different times and added to the same volume of cold 0.5 N perchloric acid. After the 30 minutes preservation in the cold, the materials were washed and resuspended in 0.1 M phosphate buffer pH 7.2. This suspension was smeared on a slide glass treated with egg albumin solution. Preparation of autoradiogram was carried out with collodion-cadmium bromide method developed by Gomberg et al. The advantage of this procedure is the good resolution obtained. In the technique of the autoradiography, the time of exposure must be determined empirically. Therefore, the standardization of the procedure is now in process to obtain a constant result.

FIG. I REVERSAL OF PH VALUE

- LEUCOMYCIN MEDIUM
- *—* STARCH MEDIUM
- SOLUBLE-STARCH MEDIUM
- STARCH MEDIUM
SPORE INOCULUM
- △—△ SOLUBLE-STARCH MEDIUM
SPORE INOCULUM

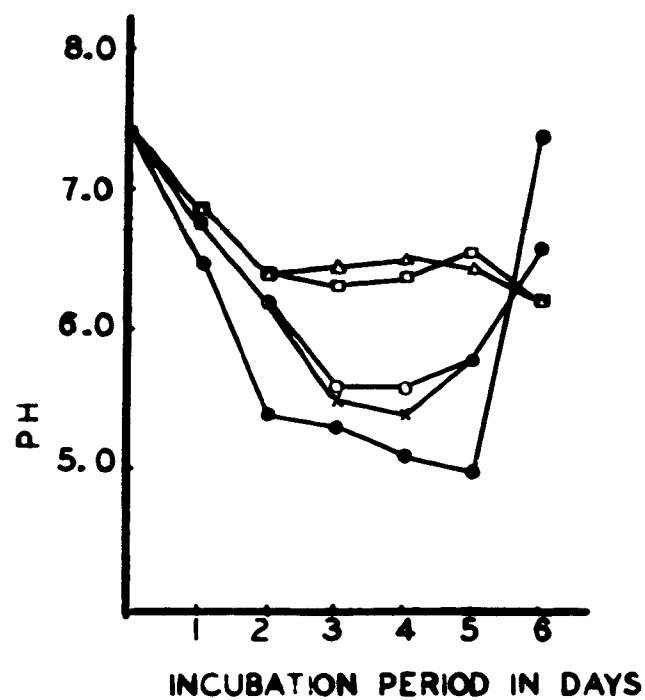


FIG.2 PRODUCTION OF LEUCOMYCIN

- LEUCOMYCIN MEDIUM
- ×—× STARCH MEDIUM
- SOLUBLE-STARCH MEDIUM
- STARCH MEDIUM
SPORE INOCULUM
- △—△ SOLUBLE-STARCH MEDIUM
SPORE INOCULUM

